In vitro combinations containing Tegobuvir are highly efficient in curing cells from HCV replicon and in delaying/preventing the development of drug resistance

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List of abbreviations: DAA: direct acting antivirals; SOC; standard of care; Peg_IFN: pegylated interferon; RBV: ribavirin; RVR: rapid virological response; cEVR: complete early virological reponse; vRVR: very rapid viral response; 2'-*C*-MeCyt: 2'-*C*-methylcytidine; TCA: Thiophene carboxylic acid; $EC_{50:}$ 50% effective concentration; $CC_{50:}$ 50 % cytotoxic concentration;

ABSTRACT

Tegobuvir (GS-9190) is a non-nucleoside inhibitor of HCV RNA replication with proven antiviral activity in HCV-infected patients. The *in vitro* antiviral activity of Tegobuvir, when combined with one or two other direct acting antivirals (DAA) was assessed. When Tegobuvir was combined with either interferon α-2b, ribavirin, the protease inhibitor (PI) VX-950, the nucleoside polymerase inhibitor (NI) 2'-*C*-methylcytidine or various non-nucleoside polymerase inhibitors, an overall additive antiviral activity was observed. Adding Tegobuvir (at concentrations of 6, 30 or 150 nM) to replicon-containing cells in the presence of suboptimal concentrations of the PI or of the various polymerase inhibitors either markedly delayed or completely prevented resistance development against these latter compounds. Tegobuvir (15 nM), when combined with the PI, was able to cure replicon-containing cells from their replicon after a single passage, whereas either compound alone (at 2-fold higher concentration) was not. The triple combination of Tegobuvir (10 nM), the PI and the NI resulted in clearance of replicon RNA after only two passages. In contrast, the inhibitors when used alone at 3-fold higher concentrations were not able to cure the cells from the replicon, after as long as 6 passages. Combinations containing low concentrations of Tegobuvir are thus highly effective in curing cells from HCV replicon and in delaying or preventing the development of resistance against other DAA.

Keywords: HCV, combination therapy, Tegobuvir, DAA, resistance development, cell curing

1. Introduction

In the past decade, the standard of care (SOC) of chronic HCV infection consisted of the combination of pegylated interferon (Peg-IFN) and ribavirin (RBV). As this therapy was only effective in 50-60% of genotype 1 infected patients and was associated with serious side effects (Manns et al., 2006), major efforts were made to develop highly potent and better tolerated inhibitors of HCV replication. Since early 2011, the NS3 protease inhibitors Telaprevir and Boceprevir have been added to the Peg-IFN /RBV regimen which significantly improved the sustained virological response. There are also a number of second generation direct acting antivirals (DAA) which are currently under clinical development (Asselah & Marcellin, 2012; Sarrazin et al., 2012). In addition, several studies in which IFN-free DAA combinations are being evaluated, with or without ribavirin, are currently on-going and some of which have produced preliminary but encouraging data. The INFORM-1 trial was the first proof-of-concept study to investigate the antiviral efficacy of an all-oral, interferon-free combination therapy, consisting of the high resistance barrier nucleoside polymerase inhibitor R7128 and NS3/4A protease inhibitor ITMN-191, in patients with chronic hepatitis C (Gane et al., 2009). In this short-term (14 days of treatment) study, no evidence of treatment-emerging resistance to either compound was identified and 99% of the patients in the treatment group had a continuous decline in viral load. The success of the INFORM-1 study demonstrated that IFN-free therapy against chronic HCV infection could become reality in the future. However, subsequent studies, in which drugs with low barriers to resistance such as an NS3/4A protease inhibitor with an NS5A inhibitor or a non-nucleoside inhibitor were combined, resulted in rather disappointing efficacies (Foster et al., 2011; Lok et al., 2012). Treatment failure caused by the emergence of viral variants carrying drug resistance mutations was frequent. Despite of the setbacks, it remains quite possible that drug resistance can be overcome by using (i) triple or quadruple DAA combinations or, (ii) combinations including at least one molecule with a high barrier to resistance.

Tegobuvir (GS-9190) is a potent and selective inhibitor of HCV genotype 1 replication. Resistance mutations (C316Y, C445F, Y448H, Y452H) are located close to the NS5B active site with C445F, Y448H and Y452H located in the β-hairpin region of NS5B (Bae et al., 2010; Shih et al., 2011). Cross-resistance analyses with the site III inhibitor A-782759 and site IV inhibitor HCV-796 revealed that Tegobuvir acts via a mechanism that is different from that of other non-nucleoside HCV

polymerase inhibitors (Shih et al., 2011). Tegobuvir undergoes a CYP 1A- mediated intracellular activation step; the resulting metabolite, after forming a glutathione conjugate, directly and specifically interacts with and inhibits NS5B (Hebner et al., 2012). When administered in combination with Peg-IFN/RBV in treatment naive GT1 patients, Tegobuvir (40mg BID) increased RVR (rapid virological response) by ~30% and cEVR (complete early virological reponse) by ~20% over Peg-IFN/RBV treatment although, no improvement in SVR was observed in the final analysis (Lawitz et al., 2011). Viral rebound and relapse were associated with the appearance of the NS5B Y448H mutant (Hebner et al., 2011). Tegobuvir, when combined with a NS3 protease inhibitor GS-9256 and Peg-IFN/RBV, resulted in 100% RVR (14 of 14 patients) in a small study (Zeuzem et al., 2012). Three additional phase II clinical trials are ongoing (GS-US-248-120, GS-US-248-0132, GS-US-248-0131) wherein the combined effect of 3 DAA combinations (the NS5A inhibitor GS-5885, the protease inhibitor GS-9451 and Tegobuvir) plus ribavirin were evaluated. Interim results indicate high on treatment response rates with the 12-24 week, IFN-free, all-oral quad regimen in HCV GT1a and 1b patients (Sulkowski et al., 2012). Despite these encouraging results development of Tegobuvir was not continued.

The rationale of the present study was (i) to assess the barrier to resistance of DAA combinations containing tegobuvir in replicon cells, (ii) to gain insight into the phenotype and genotype of potential multiple drug-resistant variants, and (iii) to assess the efficacy of triple combinations containing Tegobuvir, a nucleoside polymerase inhibitor and a NS3 protease inhibitor.

2. Materials and Methods

2.1. Cell culture

Huh 9-13 cells (Huh 7 cells containing the subgenomic HCV replicon I_{377}/NS3-3′/wt (Lohmann et al., 1999)), HuH6 cells (Huh 7 cells containing the subgenomic HCV replicon I_{389}luc-ubi-neo/NS3-3′/5.1 (Windisch et al., 2005)) or Huh 5-2 cells (Huh 7 cells carrying the subgenomic HCV replicon I_{389}luc-ubi-neo/NS3-3'/5.1 (Vrolijk et al., 2003)) were cultured as described before (Vliegen et al., 2009).

2.2. HCV Inhibitors

The HCV NS5B polymerase inhibitors 2'-*C*-MeCyt (2'-*C*-methylcytidine), JT-16 (site I, benzimidazole), TCA (site II, Thiophene carboxylic acid), HCV-796 (site IV, benzofuran) and the NS3 protease inhibitor VX-950 were synthesized according to procedures reported in publications referenced in (Paeshuyse et al., 2008). Ribavirin was purchased from ICN Pharmaceuticals (Costa Mesa, California) and recombinant IFNα 2b (intron® A) was purchased from Schering Plough (Kenilworth, NJ). The synthesis of both GS-327073 (5-[[3-(4-chlorophenyl)-5-isoxazolyl]methyl]-2-(2,3-difluorophenyl)-*5H*-imidazo[4,5-c]pyridine) and GS-9190 (5-({6-[2,4-bis(trifluoromethyl)phenyl]pyridazin-3-yl}methyl)-2-(2-fluorophenyl)-5*H*-imidazo[4,5-*c*]pyridine) has been described in the patent literature (Bondy et al., 2005, 2007, 2008a,b).

2.3. Antiviral and cytostatic assays

Antiviral and cytostatic assays were performed as described before (Paeshuyse et al., 2008). For Huh 5-2 cells, the EC_{50} was defined as the concentration of compound that reduced the firefly luciferase signal by 50 %; for Huh 9-13 and HuH6 cells as the concentration of compound that reduced the amount of HCV RNA by 50 %. The 50 % cytotoxic concentration (CC_{50}), that is defined as the concentration that inhibits the proliferation of exponentially growing cells by 50 %, was determined using the MTS/PMS method.

2.4. Combination antiviral assay

Combination antiviral assays were performed in HuH6 cells in a checkerboard format according to the protocol as described before (Vliegen et al., 2009). In HuH6 replicon-containing cells replication of the replicon is largely independent of cell proliferation, which is important when planning

combination studies with a cytostatic drug such as ribavirin (Windisch et al., 2005). The effect of drug-drug combinations were evaluated using the MacSynergy II software developed by Prichard and Shipman, analyzing data for synergism, antagonism, or additive effects (Prichard and Shipman, 1990).

2.5. Combined resistance selection

Combined resistance selection was performed using Huh 9-13 replicon-containing cells seeded in a 12-well plate as described before (Delang et al., 2009). Cells were seeded in complete DMEM containing 1 mg/ml G418 and in the presence of GS-9190 (6, 30 or 150 nM), VX-950 (3 or 6 µM), 2'-*C*-MeCyt (2 or 11 µM), JT-16 (4 or 10 µM), TCA (3 or 15 µM), HCV-796 (2 or 12 µM) or a combination of GS-9190 with either one of the other molecules in a matrix format. Following 3 weeks of selection, cultures were either fixed with ethanol and stained with 1% methylene blue or were expanded to obtain sufficient cells for subsequent phenotyping (antiviral assay) and genotypic characterization.

2.6. Clearance-rebound assay

Clearance–rebound assays were performed using Huh 9-13 cells as described before (Vliegen et al., 2009). To compare the effect of single, double and triple combinations, a total compound concentration of 25 x EC_{50} was added to the cultures [either as a sum of 8.3 x + 8.3 x +8.3 x EC_{50} or as a sum of 12.5 x + 12.5 x EC_{50} or as a single concentration of 25 x EC_{50}]. In addition, for reasons of comparison, conditions with a single concentration of 8.3 x or 25 x EC_{50} [VX-950, 2'-*C*-MeCyt or GS-9190] or 125 x EC_{50} [GS-9190] were included. In total, cells were passaged six consecutive times in the presence or absence of compound(s) and in the absence of G418. Following every passage in the presence of antiviral pressure, cells of each tissue culture flask were passaged for four consecutive times in the absence of the antiviral molecules but in the presence of 1 mg/mL G418 (rebound phase). At each passage, 3×10^5 cells were seeded in 25 cm^2 tissue culture flasks and 1.5×10^5 cells from each flask were lysed in RLT buffer. After collecting all samples, RNA was extracted using the RNeasy minikit (Qiagen, Venlo, the Netherlands) and replicon RNA content was quantified by means of RT-qPCR.

2.7. Genotypic characterization

Total cellular RNA was extracted using the RNeasy minikit (Qiagen) and was subjected to RT-PCR using primers NS3-F [5'-TTGGCTGCATCATCACTAGC-3'] (corresponding to nucleotides 1844 to 1863 of accession number AJ242652) and NS3-R [5'-TTCTCCAGTGCTGGACAGAG-3']

(corresponding to nucleotides 2822 to 2841 of accession number AJ242652) or NS5B-F [5′-TGCTTTGACTCAACGGTCAC-3′] (corresponding to nucleotides 6649 to 6668 of accession number AJ242652) and NS5B-R [5′-TGTAACCAGCAACGAACCAG-3′] (7629 to 7648 of accession number AJ242652). Nucleotide sequences were determined by automated sequencing using BigDye terminator v. 3.1 (Applied Biosystems).

3. Results

The *in vitro* antiviral activity of Tegobuvir, in combination with one or two other DAA, was evaluated in HCV replicon containing cells as follows: (i) short-term (three day) antiviral assays in which compounds were combined in checkerboard format, (ii) long-term resistance selection experiments, and (iii) clearance-rebound experiments. Tegobuvir and its close analogue GS-327073 (Vliegen et al., 2009) inhibited HCV (genotype 1) subgenomic replicon replication in Huh 9-13, Huh 5-2 and HuH6 cultures with mean EC_{50} values ranging between 1.2 – 43 nM. The in vitro anti-HCV activity of these imidazopyridines and the other molecules used in this study is summarized in Table 1 (Supplemental 1).

3.1. DAA combinations containing Tegobuvir result in an overall additive antiviral effect when evaluated in short-term (3 days) antiviral assays

Tegobuvir was combined with either the protease inhibitor VX-950, the nucleoside polymerase inhibitor 2'-*C*-MeCyt or various non-nucleoside polymerase inhibitors (benzimidazole, benzofuran and Thiophene carboxylic acid analogues) in checkerboard format and assayed in HuH6 replicon containing cells. The antiviral effect was quantified after three days of treatment with the compounds. An overall additive antiviral activity was observed (as determined by the method of Prichard and Shipman (Prichard and Shipman, 1990) (**Fig. 1. A-E**). A slightly synergistic effect was observed when Tegobuvir, at concentrations <0.05 µM, was combined with the benzofuran site IV inhibitor HCV-796 at concentrations <4.1 nM.

3.2. DAA combinations containing Tegobuvir prevent or delay the development of drug-resistant variants

When combined with the protease inhibitor VX-950 or various polymerase inhibitors (each at concentrations that were not able to clear the cells from their replicon), Tegobuvir (at concentrations of 6, 30 or 150 nM) markedly delayed or completely prevented the emergence of drug-resistant variants following 3 weeks of culturing of replicon-containing Huh 9-13 cells [**Fig. 2. A-E (i)**]. At lower concentrations of either drug in the combination, replicons with low-level resistance to both drugs emerged [**Fig. 2. A-E (ii)**]. The nucleoside analogue, 2'-*C*-MeCyt did not select for resistant variants after three weeks of culturing (**Fig. 2. B**). The combination of 5x EC_{50} of tegobuvir with 5 x EC_{50} of VX-

950 resulted in a very low number (i.e. 5) of colonies. Replicons replicating under this condition carried a signature mutation of VX-950 (A156S) and one for GS-9190 (C445F). Interestingly, VX-950 mutations that appeared in the double resistant replicons differed from those that developed under VX-950 monotherapy. The combination of 2 x EC_{50} of the benzimidazole site I non-nucleoside inhibitor JT-16 with either 5 x EC_{50} or 25 x EC_{50} of Tegobuvir (**Fig. 2. C**) resulted respectively in a very low number (i.e. 3) or no drug-resistant clones. Genotypic analysis revealed that the double resistant clones carried a Tegobuvir-resistant mutation (C445F) and a JT-16-associated mutation (T389P/S (Delang et al., 2012)). The combination of 25 x EC_{50} of site II non-nucleoside inhibitor TCA and 5 x EC_{50} of Tegobuvir (**Fig. 2. D**) completely prevented the emergence of drug-resistant variants. The combination of 25 x EC_{50} of the benzofuran site IV non-nucleoside inhibitor HCV-796 with Tegobuvir resulted in either a reduction of the number of replicating clones at lower Tegobuvir concentration (5 x EC_{50}) or a complete inhibition of viral replication at higher concentration (25 x EC_{50}). Double resistant clones were found to carry a signature mutation (C316Y) for HCV-796 and the Tegobuvir mutation C445F.

3.3. Combinations containing sub-optimal concentrations of Tegobuvir are able to completely clear hepatoma cells from HCV replicon

When used alone at 25 x EC_{50} (concentrations that were not cytostatic), each DAA was able to produce a pronounced reduction in replicon replication after one passage (4 days). Tegobuvir, at a concentration of 150 nM (125 x EC_{50}), was able to cure the cells from HCV RNA after 3 passages under the conditions used in this experiment (**Fig. 3. A**). At a lower concentration (25 x EC_{50}), Tegobuvir treatment selected for a small number of colonies that were able to proliferate (**Fig. 3. A**). Genotyping of the HCV replicon RNA isolated from the surviving cells identified a mutation at position 445 in NS5B (from cysteine to phenylalanine) that existed as a mixed population with the wild-type sequence (C445F/C), at passages C2R4 and C3R4. At later passages (C4R4 and C5R4), only phenylalanine was detected in the population. At concentrations of 12.5-fold of their respective EC_{50}, the combination of Tegobuvir (15 nM) and 2'-*C*MeCyt (5.4 µM) cured cells from their replicon after only 4 passages (**Fig. 3. B**). The combination of VX-950 (6.3 µM) and Tegobuvir (15 nM) cured cells from their replicon after only 1 passage whereas for the combination of VX-950 (6.3 µM) and 2'-*C*-MeCyt (5.4 µM) it took 2 passages to cure the cells. When Tegobuvir, at concentrations as low as 8.3-fold of its EC_{50} value (10 nM), was combined with 2'-*C*-MeCyt and VX-950 (8.3 x EC_{50}) the triple combination

was able to cure cultures from their replicon after only 1 passage. In contrast, when used alone at 3-fold higher concentrations (i.e. 25 x EC_{50}), none of the inhibitors was able to cure the cells from HCV replicon even after 6 consecutive passages in the presence of the inhibitor.

4. Discussion

We explored, using various protocols, the *in vitro* anti-HCV activity of Tegobuvir when combined with various DAAs including a protease inhibitor, a nucleoside polymerase inhibitor and 3 non-nucleoside polymerase inhibitors each interacting with a different allosteric site on the enzyme. Short term 3-day combination experiments of Tegobuvir with each DAA tested, resulted in an overall additive antiviral activity. The results from this short term combination study thus indicated that none of the combinations containing Tegobuvir resulted in an antagonistic effect and that the efficacy of such combinations could be explored further.

We here studied the effect of combinations that contain Tegobuvir on resistance development under long-term culture conditions. Our results demonstrate that Tegobuvir delayed or prevented the development of drug-resistant variants to other DAAs. This finding is important as monotherapy with most DAAs rapidly selects for resistant variants. Overall, combinations of Tegobuvir with another DAA (either a protease inhibitor, nucleoside or non-nucleoside NS5B inhibitor) had a marked effect on the emergence of drug-resistant (either mono- or double resistant) mutants. Interestingly, when double resistant mutants appeared, the mutations of such variants were not merely the sum of the mutations of the single resistant genomes. For example VX-950 alone (at 5x the EC_{50} concentration) resulted in the appearance of the V36V/A and T54T/A mutations [both described as being low- to medium-level resistance mutations (Sarrazin et al., 2007)]. In the combination VX-950 (5x EC_{50}) and Tegobuvir (5x EC_{50}) the A156S VX-950res mutation appeared. A mutation at this position leads to high-level VX-950 resistance (Sarrazin et al., 2007). Thus, suboptimal dosing of both Tegobuvir and VX-950 led to high level resistance mutations to VX-950 but low level resistance mutations to Tegobuvir. In the combination of 2'-*C*-MeCyt and Tegobuvir, despite the fact that the nucleoside only made limited contribution to replicon clearance, the observed resistance mutations to Tegobuvir changed from C445F in the mono-treatment to C316C/Y + C445F in the combination treatment. Whereas C445F resulted in low level resistance to Tegobuvir, the addition of the C316C/Y to C445F resulted in a much more pronounced resistant phenotype (Shih et al., 2011). Surprisingly, the C445C/F mutation was also observed following treatment with JT-16, a site I non-nucleoside NS5B inhibitor whose binding pocket does not involve the 445 residue. Clonal sequencing of RNA from the replicon cells revealed that the

C445F mutation pre-exists at a frequency of 3.4% (Delang et al., 2011). Moreover, replicons carrying this mutation were markedly more fit when compared to wild-type replicons (Delang et al., 2011). Altogether, these observations suggest that, in addition to result in low-level resistance to Tegobuvir, C445F also appears to increase fitness of replicon RNA in a compound independent manner and was therefore enriched along with drug-specific resistance mutations that caused a loss of replicon fitness, as in the case of the mutations to JT-16.

The ultimate goal of HCV therapy is to reduce viral replication and prevent the emergence of drug-resistant variants so that there is no relapse when therapy is stopped. We therefore compared the anti-HCV activity of Tegobuvir either alone or in combination with other DAAs in the in vitro clearance-rebound experiments. Replicon containing cells were treated with DAA combinations for extended periods of time in the absence of G418 selection. In the case that the antiviral combination was highly effective and replicon RNA was completely eradicated from the cells, the cells were no longer able to survive G418-induced killing after DAA treatment was terminated and G418 was added back to the culture medium. However, in such case that the antiviral treatment was not able to completely cure the cells from their replicon or that drug-resistant variants emerged, the hepatoma cells will be able to survive in the presence of G418. The combination containing Tegobuvir together with the protease inhibitor VX-950 and the nucleoside NS5B inhibitor 2'-*C*-MeCyt resulted in fast clearance of replicon HCV RNA. In fact, only 2 passages of combined compound pressure (each at 8.3x their EC_{50} concentrations) were needed to achieve this. Combinations with slightly higher concentrations (12.5x EC_{50} instead of 8.3x EC_{50}) of Tegobuvir and the protease inhibitor VX-950 (without the need for 2'-C-MeCyt) cleared cells from their replicon after one single passage of combined compound pressure. The clearance-rebound experiments provide support for a triple therapy containing Tegobuvir.

In summary, we have demonstrated in HCV replicon-containing cells that combinations of Tegobuvir with other DAAs are effective in curing cells from HCV replicon and in delaying or preventing the development of resistance against other DAA. These results support further development of Tegobuvir-containing IFN-free, DAA combination regimens.

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Table 1. *In vitro* antiviral activity of Tegobuvir and selected DAAs against HCV genotype 1b replicons.

Inhibitor	EC_{50} (µM)			CC_{50} (µM)
	Huh 9-13 [a]	Huh 5-2 [a]	HuH6 [a]	
Tegobuvir	0.0012 ± 0.0006	0.0054 ± 0.0018	0.043 ± 0.035	≥ 24
GS-327073	0.002 ± 0.004	0.0041 ± 0.0017	0.047 ± 0.018	≥17
2'-*C*-MeCyt	0.43 ± 0.35	3 ± 1.5	1.02 ± 0.44	≥ 26
JT-16	1.5 ± 0.83	0.80 ± 0.37	1.2 ± 0.47	22
TCA	0.12 ± 0.0067	0.29 ± 0.049	0.27 ± 0.032	>33
HCV-796	0.094 ± 0.045	0.042 ± 0.011	0.011 ± 0.0033	> 33
VX-950	0.58 ± 0.10	1.1 ± 0.71	1.4 ± 0.49	≥ 25
ribavirin	n.d.	7 ± 3	28 ± 16	> 33
IFNα 2b (IU/ml)	20 ± 9	7 ± 5	22 ± 5	>500

Data are mean values ± SD from at least 2 independent experiments
n.d.: not determined
[a] as determined by a luciferase (Huh 5-2) or RT-qPCR assay (Huh 9-13, HuH6)

Table 2: Mean volume of synergy (or antagonism) of Tegobuvir combined with:

	Tegobuvir in combination with						
	IFNα 2b	Ribavirin	VX-950	2'-*C*-MeCyt	JT-16	TCA	HCV-796
Mean volume of synergy or antagonism in μM²% (log volumes)	2 (-0.4)	4 (-0.87)	0 (-5)	6 (0)	0 (0)	1 (-3)	35 (0)

We here use a dissimilar site assumption of additivity, e.g. that two drugs affected dissimilar sites on a target enzyme or enzymes (Prichard and Shipman, 1990) . Volumes of synergy or antagonism under 25 μM²% (log volumes <2) at 95% confidence should be regarded as additive, 25-50 μM²% (log volumes >2 and <5) minor but significant amount of synergy/antagonism, 50-100 μM²% (log volumes >5 and <9) moderate synergy/antagonism and >100 μM²% (log volumes >9) strong synergy/antagonism

FIGURE LEGENDS

Fig. 1. Combined antiviral effect of Tegobuvir with a selection of HCV inhibitors in short-term (3 days) antiviral assays in replicon-containing HuH6 cells (Prichard and Shipman, 1990). The different gray scales represent different ranges of values: dark grey: −20% to 0%, light grey: 0% to 20%. Values under the zero plane indicate antagonistic activity, values in the zero plane indicate additive activity, and values above the zero plane indicate synergistic activity. All data points are averages of at least two independent experiments. Mean volumes of synergy or antagonism in brackets are presented on 95% confidence values; mean volumes between -25 and +25 indicate an additive effect– mean volumes from >25 to <50 indicate slight synergism.

Fig. 2. Combined Resistance Selection. Huh 9-13 cells were treated with Tegobuvir alone or in combination with VX-950 (A), BILN 2061 (B), JT-16 (C), HCV-796 (D) or TCA (E) at the indicated concentrations in the presence of 1 mg/ml G418. When confluent, or when a sufficiently large number of colonies had developed, cells were further passaged under the same experimental conditions. Following 5 passages, cultures were fixed (i) stained with Giemsa and (ii) phenotyped or genotyped. Published resistance mutations are marked in red. Sequences were collected for NS3 from AA 35-328 (VX-950) and NS5B from AA 242 to 540 (all combinations tested). n.d.: not determined – cells died during culture. †: cells died during combined resistance selection.

Fig. 3. Effect of Tegobuvir-containing combinations on the clearance of replicons from Huh 9-13 cells. Clearance and rebound phase data from six consecutive clearance passages [C1→C6] (in the presence of fixed concentrations of either one (A), two (B) or three compounds (C)) each followed by 4 consecutive rebound passages [R1→R4] (with G418 pressure and without compound pressure). Data are expressed as percentage of untreated controls (UTC).

Fig. 1.

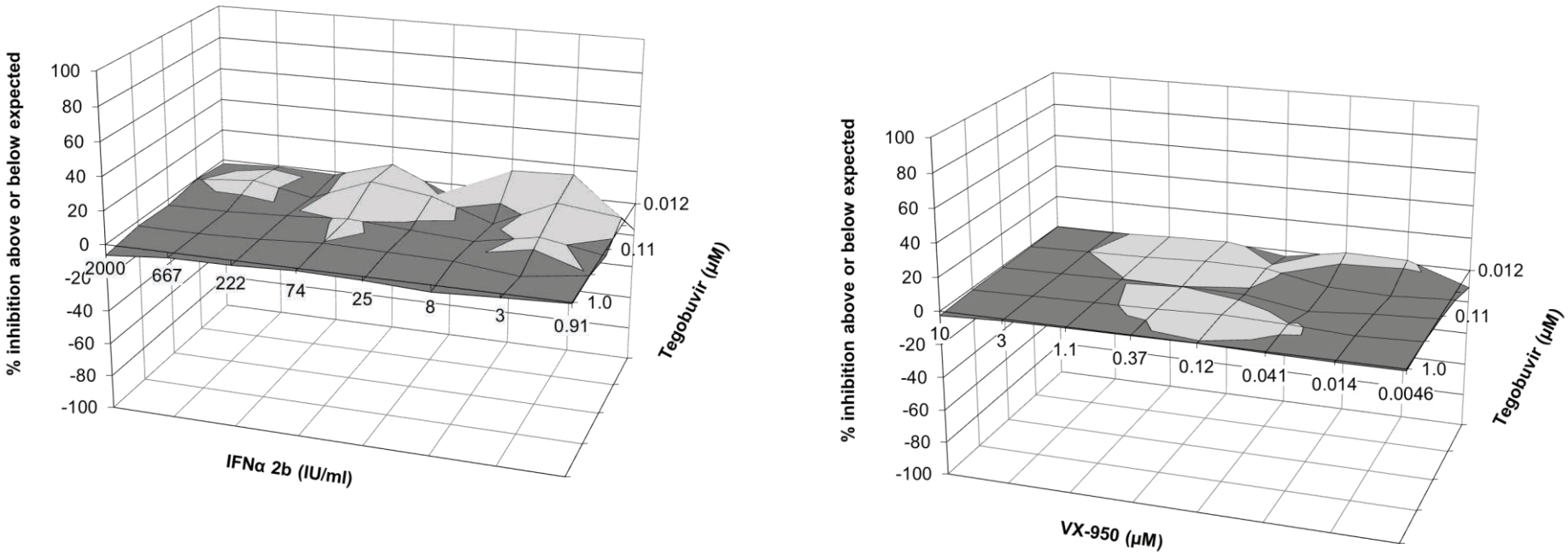
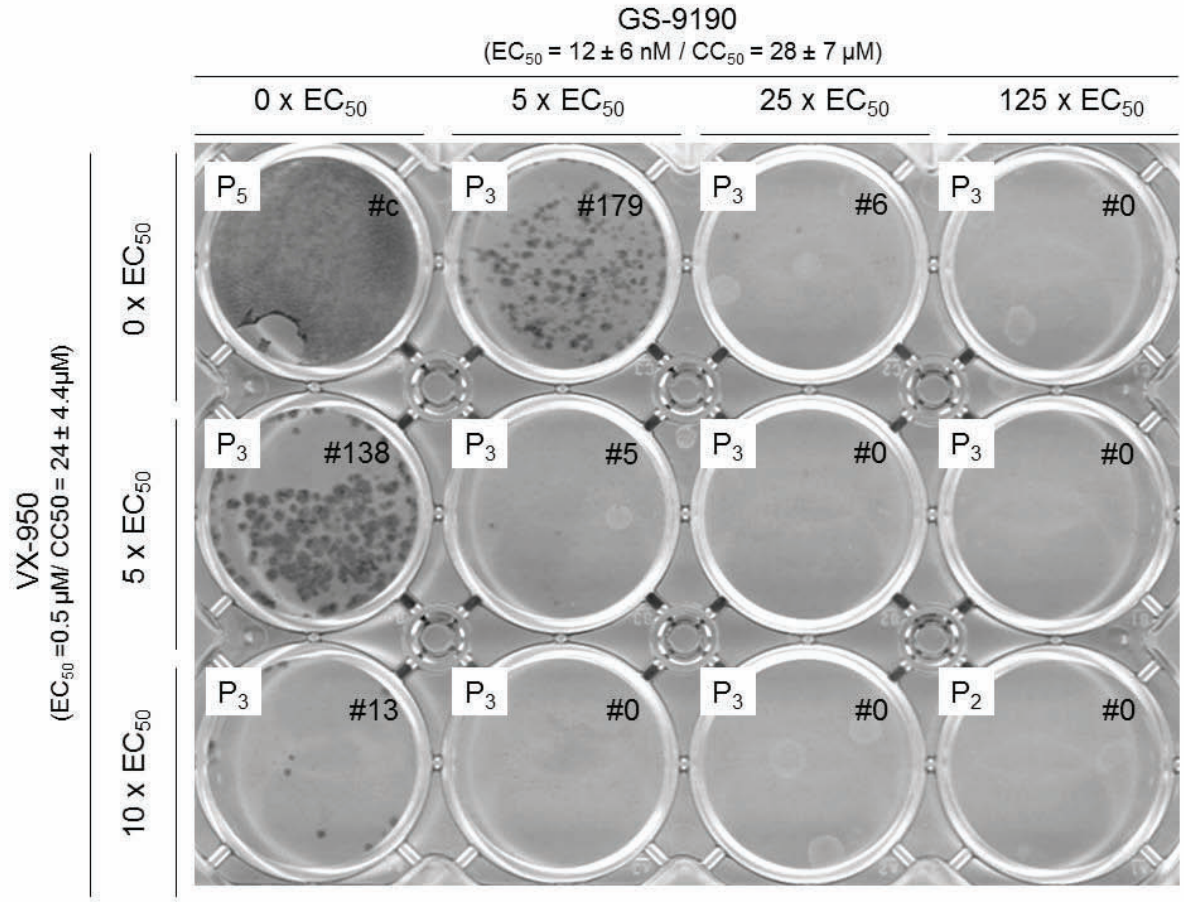

% inhibition above or below expected
100
80
60
40
20
0
-20
-40
-60
-80
-100
2000
667
222
74
25
8
3
0.91
IFNα 2b (IU/ml)
0.012
0.11
1.0
Tegobuvir (μM)
% inhibition above or below expected
100
80
60
40
20
0
-20
-40
-60
-80
-100
10
3
1.1
0.37
0.12
0.041
0.014
0.0046
VX-950 (μM)
0.012
0.11
1.0
Tegobuvir (μM)

Fig. 2.

A (i)



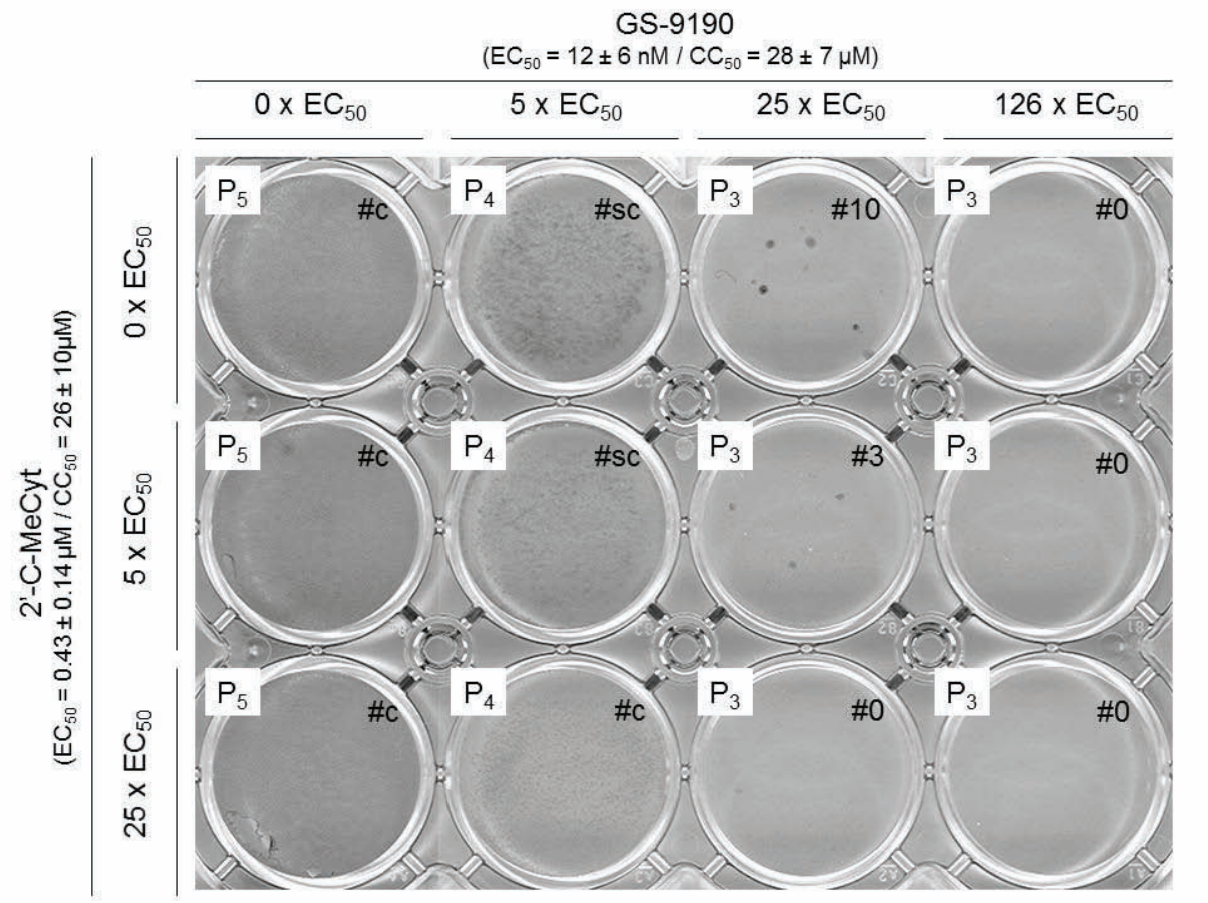
(ii)

Fold resistance when compared to 0x/0x

Tegobuvir

VX-950 \ Tegobuvir	0x EC50	5x EC50	25x EC50	125x EC50
Phenotype				
0x EC50		VX-950:1 GS-9190:**8**	n.d.	†
5x EC50	VX-950:**5** GS-9190:2	VX-950**>7** GS-9190:4	†	†
10x EC50	VX-950**≥7** GS-9190:2	†	†	†
Genotype				
0x EC50		NS3: N49N/Y NS5B: **C445F**	n.d.	†
5x EC50	NS3: **V36V/A, T54T/A** NS5B: D310N	NS3: **A156S**, T266A NS5B: L256L/P, **C445F**	†	†
10x EC50	NS3: V48V/A, **T54T/A, A156A/V** NS5B: L256L/P, R465A, T520T/P	†	†	†

B (i)



(ii)

Fold resistance when compared to 0x/0x

Tegobuvir

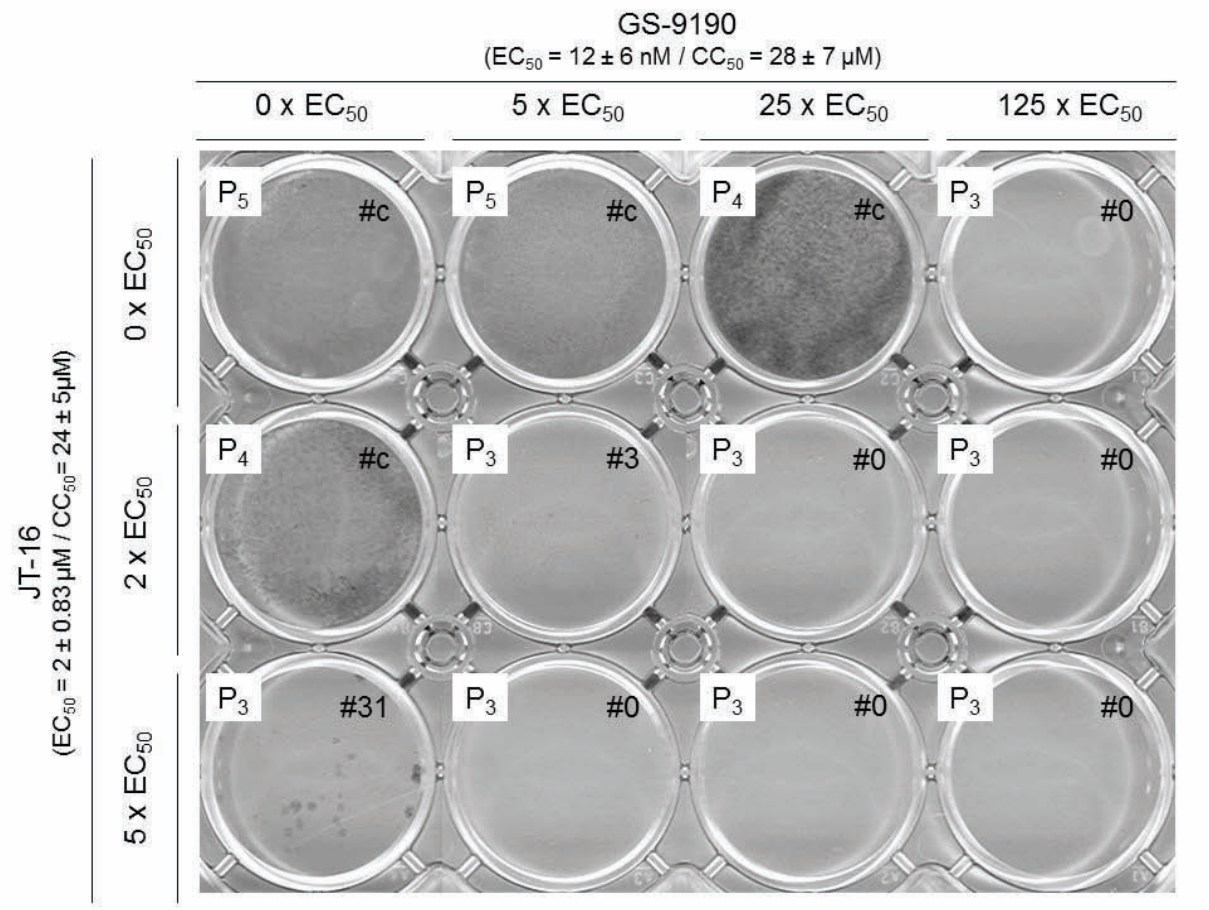
Phenotype

2'-C-MeCyt \ Tegobuvir	0x EC_{50}	5x EC_{50}	25x EC_{50}	125x EC_{50}
0x EC_{50}		2'CMC:1 GS-9190:**27**	2'CMC:1 GS-9190:**24**	†
5x EC_{50}	2'CMC:1 GS-9190:2	2'CMC:1 GS-9190:3	2'CMC:1 GS-9190:**61**	†
25x EC_{50}	2'CMC:1 GS-9190:1	2'CMC:1 GS-9190:**19**	†	†

Genotype

2'-C-MeCyt				
0x EC_{50}		NS5B: Y296Y/F, **C445F**	NS5B: Y286Y/F, **C445F**	†
5x EC_{50}	NS5B: A450A/V	NS5B: **C445F**	NS5B: Y292Y/F, **C316C/Y,** I363I/V, **C445F**, Q514Q/R	†
25x EC_{50}	NS5B: No mutation(s) detected	NS5B: **C445F**	†	†

C (i)



(ii)

Fold resistance when compared to 0x/0x

Tegobuvir

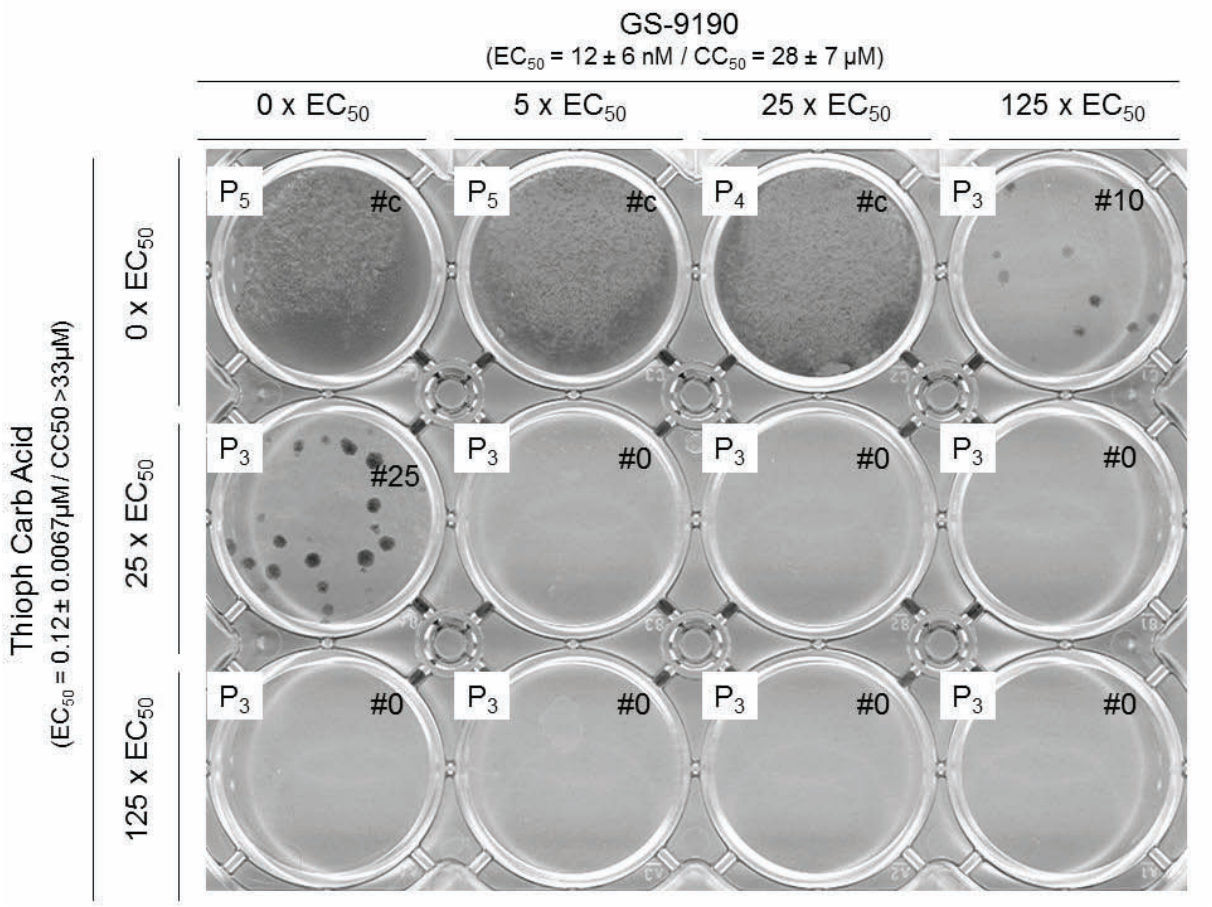
Phenotype

JT-16	0x EC50	5x EC50	25x EC50	125x EC50
0x EC50		JT-16:1 GS-9190:**4**	JT-16:1 GS-9190:**13**	†
2x EC50	JT-16:3 GS-9190: 3	JT-16:**7** GS-9190:3	†	†
5x EC50	JT-16: 2 GS-9190:1	†	†	†

Genotype

JT-16	0x EC50	5x EC50	25x EC50	125x EC50
0x EC50		NS5B: **C445C/F**	NS5B: **C445F**	†
2x EC50	NS5B: T389T/A, C445C/F	NS5B: T389P/S, **C445F**	†	†
5x EC50	NS5B: **P495A**	†	†	†

D (i)



(ii)

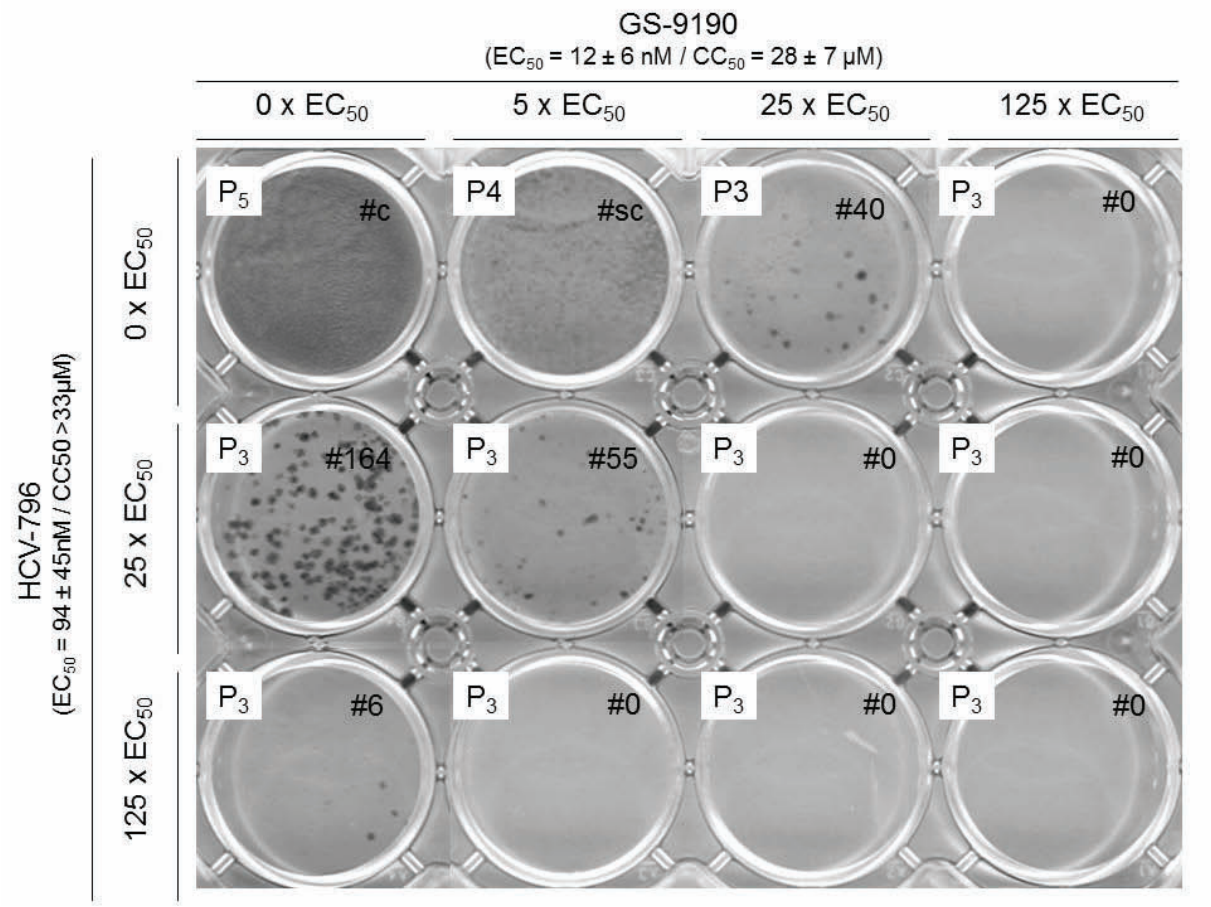
Fold resistance when compared to 0x/0x

Tegobuvir

TCA \ Phenotype	0x EC50	5x EC50	25x EC50	125x EC50
0x EC50		TCA:1 GS-9190:**4**	TCA:0.38 GS-9190:**7**	n.d.
25x EC50	TCA:**14** GS-9190: 0.31	†	†	†
125x EC50	†	†	†	†

TCA \ Genotype	0x EC50	5x EC50	25x EC50	125x EC50
0x EC50		NS5B: **C445C/F**	NS5B: **C445F**	n.d.
25x EC50	NS5B: Q251Q/P, **M423M/T**	†	†	†
125x EC50	†	†	†	†

E (i)



(ii)

Fold resistance when compared to 0x/0x

Tegobuvir

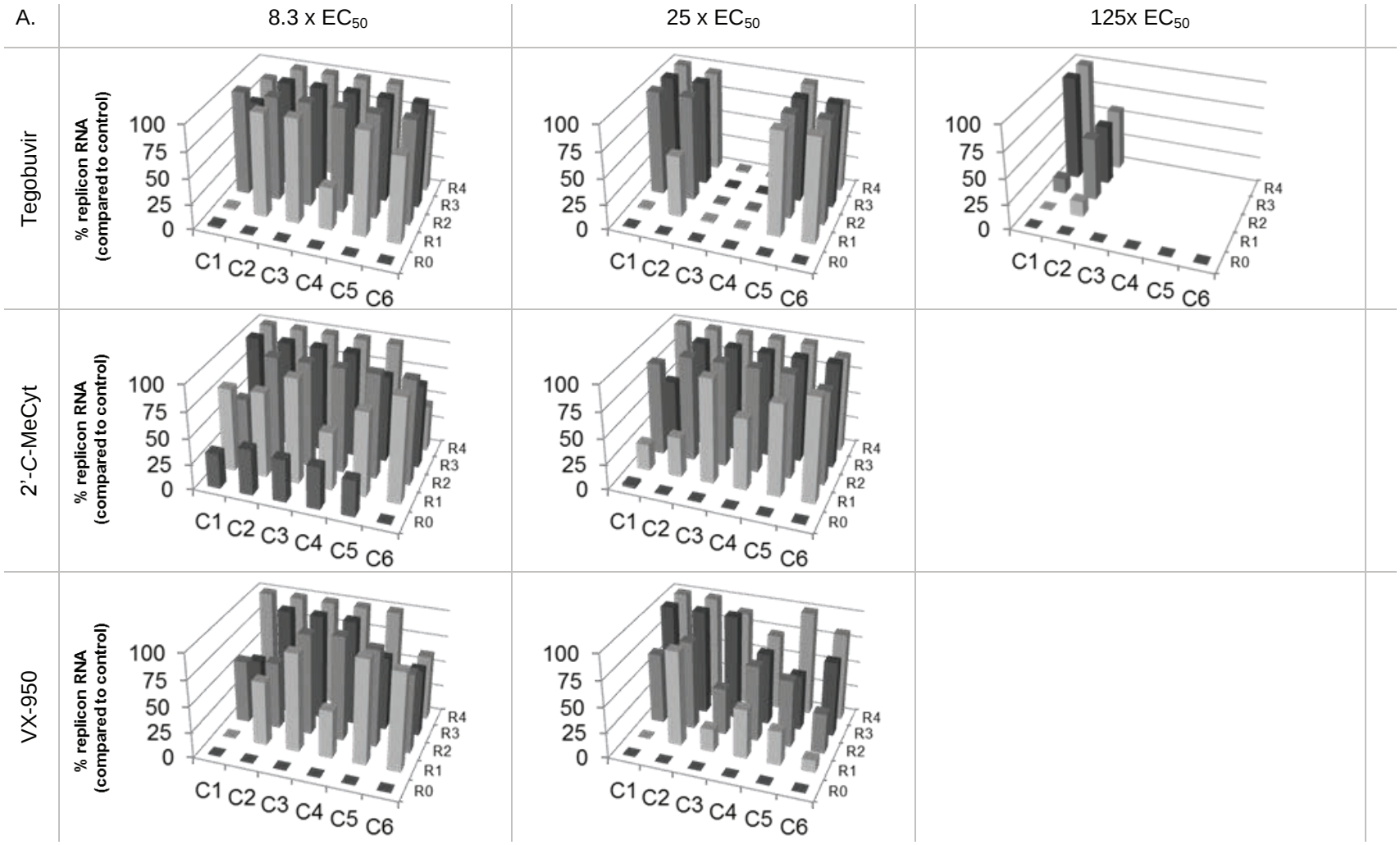
HCV-796	0x EC50	5x EC50	25x EC50	125x EC50
0x EC50		HCV-796:3 GS-9190:**9**	HCV-796:3 GS-9190:**17**	†
25x EC50	HCV-796:**157** GS-9190: **10**	HCV-796:**532** GS-9190: **33**	†	†
125x EC50	n.d.	†	†	†

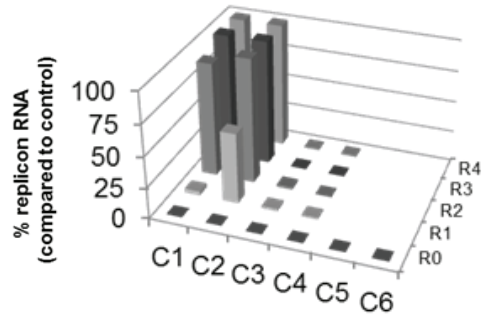
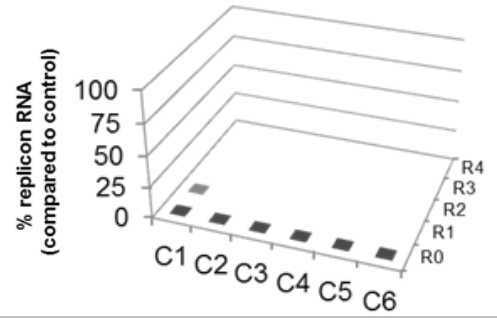
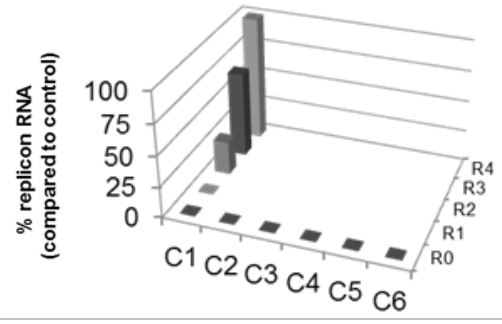
Phenotype

Genotype

HCV-796	0x EC50	5x EC50	25x EC50	125x EC50
0x EC50		NS5B: **C445C/F**	NS5B: L256L/P, D310D/A, **C445F, Y452Y/H**	†
25x EC50	NS5B: A300T/A, **C316C/Y**, **S365S/L, C445C/F**, R465V	NS5B: **C316Y**, S444D/V, **C445C/F**	†	†
125x EC50	n.d.	†	†	†

Fig. 3.


A.
8.3 x EC50
25 x EC50
125x EC50
Tegobuvir
2'-C-MeCyt
VX-950
% replicon RNA (compared to control)
100
75
50
25
0
C1 C2 C3 C4 C5 C6
R4
R3
R2
R1
R0

B.	12.5 x EC_{50} + 12.5 x EC_{50}	
Tegobuvir + 2'-C-MeCyt		
VX-950 + Tegobuvir		
VX-950 + 2'-C-MeCyt		

C.	8.3 x EC_{50} + 8.3 x EC_{50} + 8.3 x EC_{50}
Tegobuvir + 2'-C-MeCyt + VX-950